PROTECTION OF SKIN AGAINST CERCARIA PENETRATION

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FINAL PROGRESS REPORT

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I. INTRODUCTION

This project was designed to test the hypothesis that skin substantive polymers, and polymers formed within the skin can interfere with the penetration of cercaria of Schistosoma mansoni. The work reported here was closely coordinated with Dr. M. A. Stirewalt* who carried out the bioassays (cercarial penetration and worm counts). Both water-soluble and water-insoluble polymers were applied to skin (mouse tails), and low molecular weight monomers were diffused into skin and polymerized within the upper layers, to form physical or biological barriers. The results obtained indicate that application of highly charged water-soluble polymers (polyethyleneimines) can lead to substantial reduction of cercarial penetration. These materials are skin-compatible, non-toxic, non-flammable, easily applied to large areas of skin, and are commercially available products.

II. EXPERIMENTAL

A. Polysaccharide Esters

Four water-insoluble polymers (starch nonanoate, amylopectin decanoate, cellulose tridecanoate, and cellulose acetate stearate) were evaluated. These materials are soluble in chloroform, methylene chloride, and 1,1,1-trichloroethane but are insoluble in alcohols. Examination of stained mouse tails treated with solutions of these polymers indicated that at low levels of application they were not evenly coated, whereas at higher levels they became stiff and appeared to be encased in a thick sheath. Because of experimental difficulties and the potential hazards of repetitive use of halogenated organic solvents on large areas of skin, emphasis was shifted toward water-soluble, skin substantive polymers.

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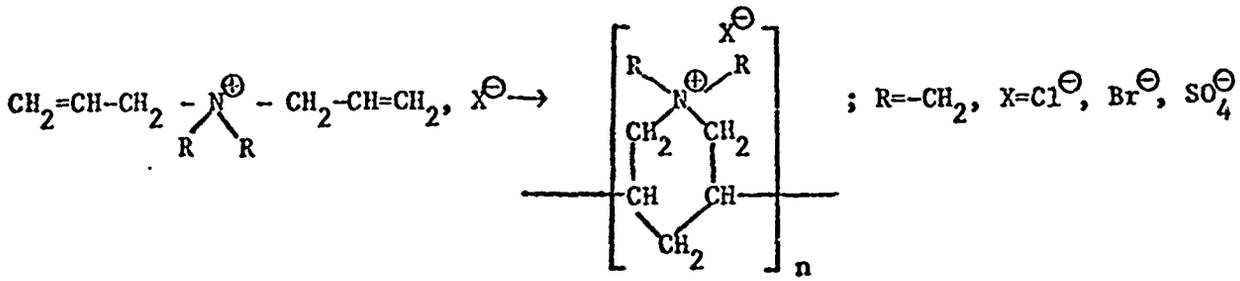
*Present address: American Foundation for Biological Research, Rockville, Maryland.

B. Water Soluble Polymers

1. CP-261

CP-261 (Calgon Company, Pittsburg, Pennsylvania) is a strongly basic water-soluble diallylamine polymer with a quaternary ring nitrogen. It sorbs to proteins and forms insoluble adducts with anionic compounds. The general structure is shown below:



Treatment of Mouse Tails

The tails were washed with ethanol to remove surface lipids and loosely adhering "dirt"; they were then air-dried and dipped into dilute solutions (0.4% to 2%) of CP-261 in distilled water (self-pH approximately 5.5) for 60 seconds and air dried. After 45 minutes the mice were either released from the holders into a carrying cage or the tails washed in distilled water for 5 minutes and again air-dried. Polymer treatments were carried out in the morning and bioassays during the afternoon of the same day.

Bioassay results are summarized in Table I expressed as the number of cercariae not penetrating (maximum = 50) per exposed mouse tail, and the number of worms found at autopsy.

2. Polyethyleneimines (PEI-18, PEI-600, PEI-1000 and PEI-600E)

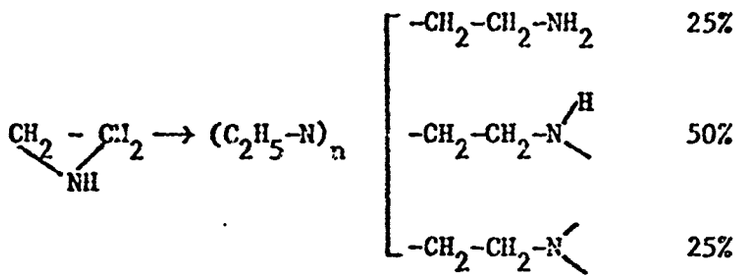
The PEI* series of polymers consists of water-soluble, branched chain condensation products of ethyleneimine, containing primary, secondary, and tertiary nitrogens:

*Dow Chemical Company, Midland, Michigan.

TABLE I

MOUSE TAIL BIOASSAY OF POLYMER CP-261

Treatment	No. of Non-penetrating Cercariae		Worms Found at Autopsy
ethanol wash; 0.4% CP-261, 60 seconds; air-dried; no water wash.	38, 42, 40, 41, 31	All cercariae dead, suspension very cloudy	None
ethanol wash; 2% CP-261, 60 seconds; air-dried; no water wash.	33, 21, 27, 40, 28		None
Controls	0, 1, 2, 2, 2		15, 11, 20, 11, 24
ethanol wash; 0.4% CP-261, 60 seconds; air-dried; no water wash.	36, 45, 46, 50	All cercariae dead, suspension very cloudy	1, 0, 0, 0, 0
ethanol wash; 0.4% CP-261, 60 seconds air-dried; 5-minute water wash.	9, 11, 17, 24, 37		10, 5, 7, 1, 0
Controls	0, 1, 1, 2, 4		26, 18, 25, 27, 17



PEI-18, PEI-600 and PEI-1000 (average molecular weights 1800, 60,000 and 100,000 respectively) are unsubstituted; PEI-600E (average molecular weight 60,000) is an ethoxylated polyethyleneimine in which almost all nitrogens are fully substituted, and which is essentially devoid of primary and secondary nitrogens.

Generally, polymer treatments were carried out by immersing each washed mouse tail (ethanol, soap and water, or water only) into 3 ml of a 0.75% solution of polyethyleneimine in 0.1% aqueous Triton X-100 for 60 seconds, and air-drying for 30 to 45 minutes. The mice were then either released from their individual holders into group carrying cages or the tails were soaked in distilled water for varying time periods, and again air dried before removal from the mouse holders. Except where indicated the bioassays were carried out on the same day as the polymer treatments, within 2 to 4 hours of treatment. The bioassay results obtained by Dr. Stirewalt, together with details of the polymer treatments are given in Tables II, III and IV.

Longevity of polyethyleneimines on mouse tail skin was estimated indirectly by staining with methylene blue followed by desorption of the dye. Methylene blue was found to stain PEI-treated mouse tails, but not untreated tails. Desorption experiments were carried out by the following procedure: isolated mouse tails were washed in ethanol, air dried, dipped into 0.75% PEI (in 0.1% Triton X-100) either at self-pH (9.5) or at pH 7.0 for 60 seconds, and again air-dried. They were then either immersed directly in 0.5% aqueous methylene blue for one minute or soaked in distilled water for varying time periods before dye treatment. After removal from the dye bath, the mouse tails were rinsed free of excess dye. Bound methylene blue

TABLE IIa

MOUSE TAIL BIOASSAY OF PEI-18

Treatment*	No. of Non-penetrating Cercariae per mouse (maximum = 50)	Worms Found at Autopsy
60 seconds dip into 0.75% PEI-18 in 0.1% Triton X-100; and air-dried	35 (n=27)	1/27 mice
60 seconds dip into 0.75% PEI-18 in 0.1% Triton X-100; air dried, retreated for 60 seconds and air dried	34 (n=6)	1/6 mice
no polymer; 60 seconds in 0.1% Triton X-100 then air dried (n=3)	13 (n=3)	17/3 mice (6 per mouse)
Controls	2 (n=18)	368/17 mice (22 per mouse)

*All mouse tails, except controls, pre-washed with absolute ethanol and air-dried.

TABLE IIb

MOUSE TAIL BIOASSAY OF PEI-18

Treatment*	No. of Non-penetrating Cercariae per mouse (maximum = 50)
5% PEI-18 in 0.1% Triton X-100	
30 seconds wash post-treatment	33.3 (n=5)
30 minutes wash post-treatment	18.5 (n=5)
2% PEI-18 in 0.1% Triton X-100	
30 seconds wash post-treatment	32 0 (n=4)
30 minutes wash post-treatment	15.2 (n=5)
Untreated controls	1.6 (n=10)

*All mouse tails pre-washed by 30 second rinse in distilled water; exposure to polymer solutions, 60 seconds; followed by 30-minute air drying before wash in distilled water; no autopsies performed.

TABLE IIIa

MOUSE TAIL BIOASSAY OF PEI-600

Treatment*	No. of Non-penetrating Cercariae per mouse (maximum = 50)	Worms Found at Autopsy
Self-pH (9.5) (in 0.1% Triton X-100)		
no wash	40 (n=5)	None
30 seconds wash	25 (n=5)	None
5 minutes wash	31 (n=5)	3/5 mice
30 minutes wash	22 (n=5)	15/5 mice (3 per mouse)
pH 7.0, in distilled water		
30 seconds wash	30 (n=5)	1/5 mice
30 minutes wash	14 (n=5)	50/5 mice (10 per mouse)
Untreated controls	2.3 (n=15)	354/14 mice (~25 per mouse)

*All mouse tails, except controls, pre-washed with absolute ethanol; polymer concentration: 0.75%; Treatment times: 60 seconds, then air-dried for 30 minutes before washing with distilled water.

TABLE IIIb

EFFECTS OF PRE-WASH ON PROTECTION BY PEI-600

Treatment* (Pre-wash)	No. of Non-penetrating Cercariae per mouse (maximum = 50)	Worms Found at Autopsy
Ethanol	27 (n=10)	3/10 mice
Distilled water	26 (n=20)	1/20 mice
Soap and water, then rinsed	28 (n=10)	10/10 mice (1 per mouse)
Unwashed controls (no polymer)	1.5 (n=20)	439/18 mice (24 per mouse)

*Polymer concentration: 0.75% in 0.1% Triton X-100; Treatment time: 60 seconds; air dried 30 minutes, then washed in distilled water for 30 seconds. Assayed on day of treatment.

TABLE IIIc

LONGEVITY OF PROTECTION BY PEI-600*

Treatment	No. of Non-penetrating Cercariae per mouse (maximum = 50)	Worms Found at Autopsy
Treated and exposed 26 hours later	17 (n=20)	220/20 mice (11 per mouse)
Controls	1.5 (n=10)	299/10 mice (~30 per mouse)

*Polymer concentration: 0.75% in 0.1% Triton X-100; Treatment time: 60 seconds; air dried 45 minutes then washed in distilled water 30 seconds. Exposed to cercariae the next day.

TABLE IV

MOUSE TAIL BIOASSAY OF PEI-600E AND PEI-1000

Treatment*	No. of Non-penetrating Cercariae per mouse (maximum = 50)	Worms Found at Autopsy
PEI-600E no post-treatment wash	40 (n=5)	None
PE-600E 30 minute post-treatment wash	20 (n=5)	44/5 mice (~9 per mouse)
PEI-1000 no post-treatment wash	38 (n=5)	None
PEI-1000 30 minute post-treatment wash	31 (n=5)	6/5 mice (~1 per mouse)
Controls	3.4 (n=10)	200/10 mice (20 per mouse)
Ethanol wash only	4 (n=6)	158/6 mice (~26 per mouse)
Ethanol wash plus 0.1% Triton X-100 (no polymer)	15 (n=6)	76/6 mice (~12 per mouse)

*All mouse tails, except controls, pre-washed with absolute ethanol; polymer concentrations, 0.75% in 0.1% Triton X-100; 60 seconds treatment followed by 30 minutes air-drying before wash in distilled water.

was then removed by extracting each tail with 0.1N H_2SO_4. Absorbances of the acid extracts were measured at 660 nm. Typical results with PEI-18 and PEI-600 are shown in Figure 1.

Large amounts of polymer appear to be washed off during the first minute, and considerably less during the next 30 to 60 minutes. The absolute amounts of polymer sorbed to the skin were not measured; however, bioassay data indicate residual activity after 30 minutes soaking in water. In the case of PEI-600 less polymer appeared to be sorbed at pH 7.0 than at pH 9.5. These data parallel the bioassay experiments (Table IIIa) in which protection against cercaria penetration was better at the higher pH.

C. "Internal Polymerization" of Acrylates

The deposition of polymers within the skin was carried out by procedures previously developed in-house, using both isolated guinea pig stratum corneum, and intact animals. The procedure is based on the finding* that the combination of an oxygen scavenger (THPC, tetrakis(hydroxymethyl)phosphonium chloride) with persulfates and either methacrylic acid or methacrylamide results in polymer formation within the upper layers of skin without the exclusion of oxygen. The reaction is rapid, going essentially to completion in 20 to 30 minutes.

In vivo "internal polymerization" was carried out as follows: The reaction mixture (complete system) contained 5.0g methacrylic acid (MAA) or 5.0g methacrylamide (MAM), 1.2g ammonium persulfate, and 0.72g tetrakis (hydroxymethyl) phosphonium chloride (THPC) in 100 ml of 0.01N H_2SO_4. Individual mouse tails were soaked for 30 minutes in 10 ml of reaction mixture, rinsed twice with distilled water, blotted with filter paper and left to air dry for 15 minutes. The treated tails were whitish and somewhat stiff. To prevent injury (the mice tended to bite each other's tails), the animals were transported in small individual cages, rather than in group

*A. D. Jenkins and L. J. Wolfram, "Vinyl Polymerization in the Presence of Phosphine Compounds as Oxygen Removers," U. S. Patent No. 3,248,376.

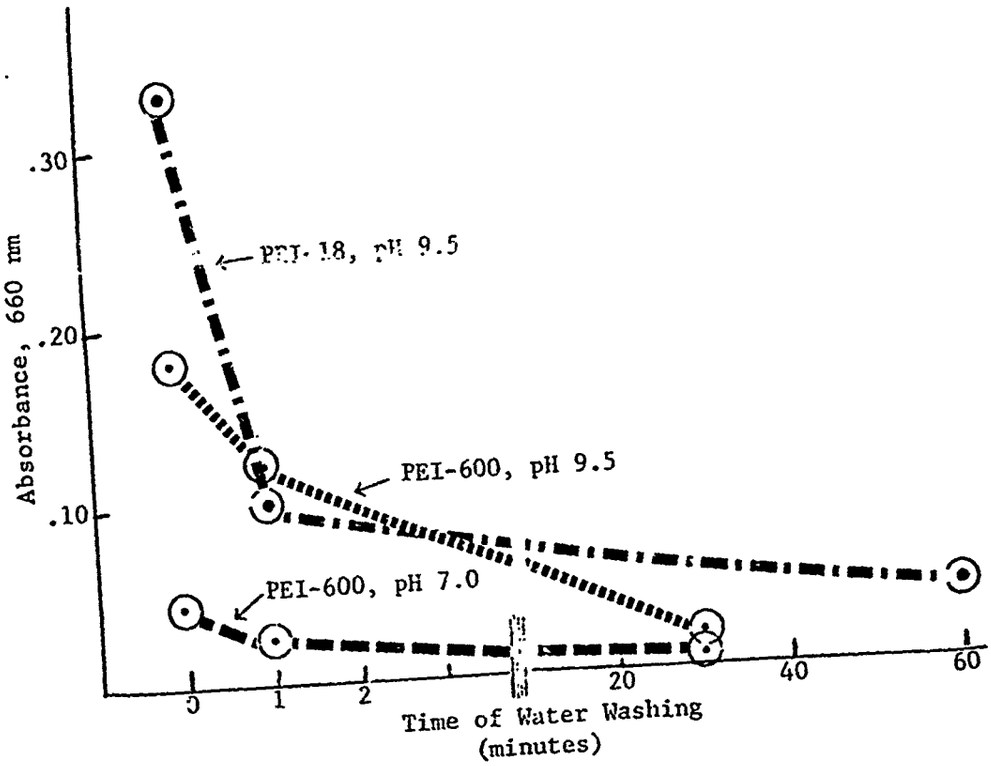


Figure 1. Dye Take-up by Residual PEI on Washed Mouse Tails.

carrying cages. They were exposed to cercarial infection on the day of treatment. The penetration data, summarized in Table V, indicated only little, if any, protection.

III. DISCUSSION OF RESULTS

The data obtained with water-soluble cationic polymers, especially with the polyethyleneimines, indicate a considerable level of protection against cercaria penetration when exposure takes place on the day of treatment. Although some cercariae appeared to penetrate, the worm burden at autopsy was very low.

When PEI-600 was applied from an unbuffered solution (self-pH, about 9.5) a total of 14 worms were found in 40 mice (Table IIIb). When treated mouse tails were soaked in water for 30 minutes, dried, and then assayed, three worms per mouse were found at autopsy (Table IIIa), whereas untreated controls had worm burdens of 20 to 30 per animal. After 24 hours the effectiveness of PEI-600 decreased to a low level. Thus, the major problem at present is to increase the longevity of the polymer on skin. This might be accomplished by using polymers of higher molecular weight, by crosslinking after deposition, or by chemical modification of the polymer (introduction of functional groups) which would increase skin substantivity and decrease water solubility. Some of the advantages of polyethyleneimines, as compared to other potential protective systems, are ease of application from a simple aqueous solution, skin compatibility, and commercial availability.

The other approaches tested in the course of this program, use of polysaccharide esters of fatty acids and "internal polymerization" of acrylates showed considerably less promise than did the water-soluble polymers. Use of the polysaccharide esters as physical barriers was impractical since they

either gave incomplete coverage or thick, rigid films. Diffusion of monomers into the skin followed by polymerization offered essentially no protection against cercarial penetration.

V. R. Usdin
Principal Investigator

Other personnel: A. Waldman

TABLE V

EFFECTS OF "INTERNAL POLYMERIZATION" OF ACRYLATES

Treatment	No. of Non-penetrating Cercariae per mouse (maximum = 50)
MAA* without catalyst ("blank")	6.4 (n=5)
MAA with catalyst (complete system)	11.2 (n=5)
Untreated controls	1.0 (n=5)
MAM** without catalyst ("blank")	2.7 (n=10)
MAM with catalyst (complete system)	1.5 (n=10)
Untreated controls	0.6 (n=10)

*MAA = methacrylic acid

**MAM = methacrylamide